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Structure-based rational design of a novel chimeric PD1-NKG2D receptor for natural killer cells

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ABSTRACT

Chimeric antigen receptor (CAR)-engineered natural killer (NK) cells have the potential to provide the potential for the implementation of allogeneic "off-the-shelf" cellular therapy against cancers. Currently, most CARs are not optimized for NK cells, so new NK-tailored CARs are needed. Here, a major activating receptor of NK cells, NKG2D was harnessed to design different chimeric receptors that mediate strong NK cell signaling. In these NKG2D signaling-based chimeric receptors, the extracellular domain of inhibitory receptor PD-1 was employed to reverse the immune escape mediated by PD-1 ligands in the solid tumors. To achieve the rational design of chimeric PD1-NKG2D receptors, we developed a transmembrane protein tertiary structure prediction program (PredMP & I-TASSER) and optimized the conformation of the PD-1 ectodomain by genetically altering the sequences encoding the hinge and intracellular domain. Finally, we identified a chimeric PD1-NKG2D receptor containing NKG2D hinge region and 4-1BB co-stimulatory domain to exhibit stable surface expression and mediate *in vitro* cytotoxicity of NK92 cells against various tumor cells. This strategy now provides a promising approach for the computer-aided design (CAD) of potent NK cell-tailored chimeric receptors with NKG2D signaling.

1. Introduction

Adoptive cellular immunotherapy has become a promising clinical treatment for malignancies, such as chimeric antigen receptor (CAR) T cells have shown potency against hematopoietic tumors (June et al., 2018). Due to the necessary collection and gene modification of each patient's T cells, autologous cell-based CAR-T therapies are costly and labor-intensive and thus sometimes impact the treatment of patients (Jung and Lee, 2018). Natural killer (NK) cells play a central role in the defense against viral infections and cancer (Guillerey et al., 2016; Morvan and Lanier, 2016). NK cells are one of the alternatives to T cells in CAR-based therapies. CAR-NK cells do not present the same side effects such as cytokine release syndrome (CRS) in CAR-T therapy (Rezvani et al., 2017). The development of CAR-NK immunotherapy is still in early stages, mainly because of difficulty in the large-scale expansion of NK cells. To solve this problem, continuously expanding human NK cell lines (*e.g.* NK92 cells) (Williams et al., 2017) and iPSC-derived NK cells (Li et al., 2018) have been established. NK cells with cytotoxic activity against malignant cells and minimal side effects deserve to function as ideal allogeneic effectors to provide an "off-the-shelf" cellular immunotherapy (Zhang et al., 2017).

Most CAR components, especially the signaling domains, are derived from T cell receptor signaling moieties and need to be optimized for NK cell signaling (Siegler et al., 2018). The incorporation of NK cell activating receptors may lead to cytotoxicity enhancement of CAR-NK cells. Activating receptors in NK cells include natural cytotoxic receptors (NCRs) and co-stimulatory receptors. In human, NKG2D is a key activation receptor for NK cells to eliminates stressed, malignant transformed, and infected cells (Lanier, 2015; Sheppard et al., 2018; Stojanovic et al., 2018), thus it provides excellent potential for NK-tailored CAR construction (Sentman and Meehan, 2014). NKG2D lacks signaling motif in its cytoplasmic domain and can deliver signals only in association with its transmembrane signaling adaptor proteins DAP10 (Garrity et al., 2005). Unfortunately, NKG2D is a type II transmembrane protein with an orientation opposite to current CAR platforms, thus the adoption of NKG2D transmembrane domain will alter the configuration and function of other CAR elements. Due to these reasons and the intrinsic instability (Park et al., 2011) of NKG2D protein, the use of

Abbreviations: CAR, chimeric antigen receptor; EC, extracellular; TM, transmembrane; CP, cytoplasmic

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NKG2D transmembrane domain in CARs to mediate NK cell activation still remains challenging so far. Furthermore, to optimize of CAR signaling with NKG2D in NK cells, it is necessary to incorporate costimulatory domain of 4-1BB (CD137). Costimulatory molecule 4-1BB can regulates NKG2D costimulation in human (Kim et al., 2008) and improve NK cell-based CAR functionality (activation, proliferation, and persistence) through the activation of downstream signaling pathway (Oberschmidt et al., 2017).

Here we rationally designed a series of chimeric PD1-NKG2D (chPN) receptors mainly based on the tertiary structure prediction to utilize the natural NKG2D signaling to induce robust cytotoxicity activity of NK92 cells. The backbone of these chimeric receptors was constructed by fusing the ectodomain of PD-1 to the transmembrane domain of NKG2D. These chPN receptors intended to switch the negative PD-1 signal to an activating signal and hence reversed the immune suppressive effects of PD-1 upon interaction with its ligands in solid tumors (Liu et al., 2016). Importantly, we demonstrated for the first time how to reduce the interference of the NKG2D transmembrane domain on the conformation and function of flanking regions in chimeric receptors *via* 3D structure modeling of transmembrane proteins. Finally, NK92 cells engineered to express the chPN receptor with NKG2D hinge region and 4-1BB cytoplasmic domain exhibited stable surface expression and robust *in vitro* cytotoxicity against human lung cancer cells.

2. Materials and methods

2.1. Cell lines and cell culture

Human NK92 cell line, human HEK293 T cell line, human lung carcinoma cell lines A549, H1299, human melanoma cell line SK-MEL-28, human bone osteosarcoma epithelial cell line U2OS, and murine mammary carcinoma cell line EMT6 were obtained from American Type Culture Collection (Manassas, USA). NK92 cells were cultured in minimum essential medium alpha (α-MEM) containing heat-inactivated 12.5% horse serum (Solarbio, China), 12.5% heat-inactivated fetal bovine serum (FBS) (Biological Industries, Beit Haemek, Israel), 100 U/mL penicillin, 100 μg/mL streptomycin (Beyotime Biotechnology, Shanghai, China), and 200 U/ml recombinant human IL-2 (Peprotech, USA). HEK293 T and A549 cells were cultured in DMEM (Hyclone, USA) supplemented with 10% heat-inactive FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. H1299 cells, SK-MEL-28 cells, U2OS cells and EMT6 cells were cultured in RPMI 1640 (Hyclone, USA) containing the same supplements as for DMEM.

2.2. Construction of chimeric PD1-NKG2D (chPN) receptors

Four PD1-NKG2D-based chimeric receptors were constructed. All cDNAs were derived from *de novo* gene synthesis (Tianyi Huiyuan Biological Technology Co., Ltd, China). Basic PN receptor was constructed by fusing hinge region [aa 73–90], transmembrane and cytoplasmic domains [aa 1–72] of human NKG2D to human PD-1 extracellular domain [aa 21–170]. PNBB was constructed by fusing PN to the human 4-1BB cytoplasmic domain [aa 214–255]. PLNBB was constructed by inserting a flexible $(GGGGS)_3$ linker between the NKG2D hinge region and the PD-1 ectodomain of PNBB. The GS linker 15 amino acids in length. All constructs were further cloned into pCDH-CMV-MCS-EF1α-copGFP vector (System Biosciences, Palo Alto, USA) with standard molecular biology techniques. The PNBBD vector was constructed by replacing copGFP in the PNBB expressing vector with full-length human DAP10.

2.3. Transmembrane protein structure modeling

For transmembrane proteins, PD-1 and chimeric PD1-NKG2D receptors, the 3D structure models of transmembrane and extracellular domains were built by use of PredMP sever (http://predmp.com) and I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). First, the chimeric receptor structure was predicted using PredMP server to obtain the precise conformation of the transmembrane domain. The structure of the extracellular domain was then remodeled and refined by using the I-TASSER server. Finally, the 3D structural model of each chimeric receptor was established by combining the above two prediction models.

2.4. Lentivirus production and transduction

Lentiviral constructs pCDH-CMV-MCS-EF1-copGFP or pCDH-CMV-chPN were co-transfected with packaging plasmid psPAX2 and pMD2.G into HEK293 T cells using Calcium Phosphate Cell Transfection Kit (Beyotime Biotechnology, Shanghai, China). Virus particles were harvested and concentrated by incubation with PEG 8000 (80 g/L) and NaCl (17.5 g/L) at 48 h and 72 h post-transfection. NK92 cells were infected with the chPNs-expressing lentivirus or control lentivirus in the presence of 8 μg/mL polybrene, and the resulting cell lines were named PN-NK92, PNBB-NK92, PLNBB-NK92, and PNBBD-NK92, respectively.

2.5. Quantitative real-time PCR (qPCR)

Total RNA of suspension-cultured NK92 cells and engineered NK92 cells was isolated using the RNAiso Plus (Takara Bio Inc., Kusatsu, Japan). mRNA was transcribed into cDNA using OneScript Plus Reverse Transcriptase (Applied Biological Materials, China). q-PCR was performed using EvaGreen 2x qPCR MasterMix (Applied Biological Materials, China) on the LightCycler 480 II System (Roche, Basel, Switzerland). Primer sequences are as follows:

chPN: 5′- GTGTCACACAACTGCCCAAC -3′ and 5′- CCGCAGGCTCT CTTTGATCT -3′.

GAPDH: 5′-GAGGACCTGACCTGCCGTCT-3′ and 5′-GGAGGAGTGG GTGTCGCTGT-3′.

The amount of PCR product for chPN was compared with the amount of GAPDH product to estimate the amount of variation between samples.

2.6. Flow cytometry

Analysis of PD-1 and NKG2D expression on chPN-engineered NK92 cells was done by staining with APC-conjugated anti-mouse PD-1 mAbs (BD Biosciences, USA) and APC-conjugated anti-mouse NKG2D mAbs (eBioscience, USA) at 96 h post-transfection. PD-L1 expression in each cancer cell line was stained with APC-conjugated anti-mouse PD-L1 mAbs (BD Biosciences, USA). Samples were analyzed by Guava® easyCyte™ Flow Cytometer (Millipore, USA). Flow cytometry data were analyzed by FlowJo V10 software (FlowJo LLC, Ashland, USA).

2.7. Cytotoxicity assay

Cytotoxicity of chPN-engineered NK92 cells was determined by LDH release assay. LDH release assays were done using the LDH Cytotoxicity Assay Kit (Beyotime Biotechnology, Shanghai, China) following the manufacturer's protocol. Untranduced or tranduced NK92 cells and tumor cells were co-cultured in α-MEM medium at different effector to target ratios (E:T ratios) of 1:1, 2.5:1 and 5:1 in triplicates in 96-well flat-bottom plates for 5 h. The percentage of specific lysis at each E:T ratio was calculated.

2.8. Cytokine assay

Untranduced or tranduced NK92 cells were co-cultured with tumor cells in α-MEM complete medium at an E:T ratio 5:1 in triplicate wells. Culture supernatant was harvested after 24 h, and the concentrations of IFN-γ, TNF-α, Granzyme B and perforin were determined by ELISA kits according to the manufacturer's instructions (ABclonal, Biolegend and

Abcam).

2.9. High content cell imaging

H1299 cells were co-cultured with untransduced NK92 cells or PNBB-NK92 cells at the E:T ratio of 5:1 in a 96-well plate. After 1.5-h incubation, the plate was were scanned and images collected with the high content cell imaging system at 20× magnification with a photo interval of 2 min. taking photos for a total of 4.5 h. The photo taking process lasted for 4.5 h.

2.10. Statistical analysis

Unpaired two-tailed Student's *t*-test was used to compare two group means, as indicated in each figure legend. By convention, we referred to statistically significant as $P < 0.05$ and statistically highly significant as $P < 0.001$. Statistical analysis was performed with GraphPad Prism software (GraphPad, La Jolla, USA).

3. Results

3.1. Structure-based design and construction of chimeric PD1-NKG2D receptors

The orientation of chimeric PD1-NKG2D (chPN) receptors was determined by the type II transmembrane protein NKG2D, and fusion to NKG2D transmembrane domain would induce conformational changes in the ectodomain of the type I protein PD-1. To precisely assess and optimize the conformation of PD-1 ectodomain in the chimeric receptors, we developed a new method for modeling transmembrane protein structure. The structure modeling was based on the crystal structure of human PD-1/PD-L1 complex (Pascolutti et al., 2016). To facilitate the binding of PD-1 to its ligands, the conformation of the PD-1 ectodomain in each chPN receptor is required to be close to its native conformation in wild-type PD-1 protein.

By using the two protein structure modeling servers of PredMP (Wang et al., 2019) and ITASSER (Yang et al., 2015) in combination, the 3D structure of extracellular and transmembrane domains of the wild-type PD-1 and chPN receptors was successfully constructed (Fig. 1A). When PD-1 ectodomain was directly fused to NKG2D transmembrane domain, the ectodomain of the chimeric receptor PN0 lacked conformational flexibility due to a shorter distance to the membrane and a shorter random coil compared to wild-type PD-1. While the N-terminal 18-amino acid segment (membrane proximal hinge region) of NKG2D ectodomain remained, the chimeric receptor PN possessed a more flexible ectodomain conformation. Furthermore, the insertion of a flexible $(GGGGS)_3$ linker between PD1 ectodomain and NKG2D hinge region further provided ectodomain of the chimeric receptor PLN with a conformation closest to wild-type PD-1. These results indicated that the chimeric receptors PN and PLN with the near-native conformation of PD-1 ectodomain were suitable as the basis for the construction of chPN receptors.

We next constructed four different chPN receptors with PN or PLN as the backbone (Fig. 1B). Among them, the chimeric receptor PN was directly constructed according to PN in Fig. 1A. To improve the proliferation and persistence of NK cells and the stability of chimeric proteins, chimeric receptors PNBB and PLNBB were constructed by fusing 4-1BB cytoplasmic domain to the PN and PLN, respectively. In addition, another chimeric receptor, PNBBD was constructed on the basis of PNBB by exogenous expression of the adaptor protein DAP10 to simulate the natural signaling mode of the NKG2D-DAP10 axis (Chang et al., 2013), instead of fusing the DAP10 intracellular domain to the N-terminus of NKG2D like the 4-1BB intracellular domain. According to the prediction of transmembrane helices (data not shown), all four chPN receptors were localized to the cell membrane *via* the NKG2D transmembrane domain, while each C-terminal PD-1 ectodomain was located outside the membrane (Fig. 1C).

3.2. Expression profiles of chimeric PD1-NKG2D receptor-engineered NK92 cells

To generate NK92 cells expressing the chPN receptor, NK92 cells were infected with lentivirus expressing each pCDH-chPN construct (Fig. 1B). The infected cells were denoted as PN-NK92, PNBB-NK92, PLNBB-NK92, and PNBBD-NK92, respectively. Analysis of chPN mRNA expression in each engineered NK92 cells was performed by using qPCR. The relative expression of chPN receptors showed that chimeric receptor expression was significantly higher in all engineered NK92 cells than in the untransduced (WT) NK92 cells (Fig. 2A). Meanwhile, PNBB and PLNBB exhibited the highest expression level, compared to PN and PNBBD (PNBB ˜ PLNBB > PN > PNBBD). In this case, the difference in transcriptional expression of various chPN receptors in NK92 cells likely reflects transcription interference (TI) generated by tandem promoters (Palmer et al., 2011) (pCMV and pEF1α) in pCDH vector.

We next confirmed the transduction efficiency of each chPN receptor by detecting enhanced green fluorescence (EGFP). The results showed that engineered NK92 cells had much higher levels of EGFP expression compared to the untransduced NK92 cells, with the exception of PNBBD, in which the EGFP gene was replaced by DAP10 (Fig. 2B, Table S1). Surface expression of PD-1 ectodomain on untransduced and chPN receptor-engineered NK92 cells was assessed by flow cytometry. Interestingly, compared to untransduced NK92 cells, only PNBB-NK92 cells exhibited significantly increased PD-1

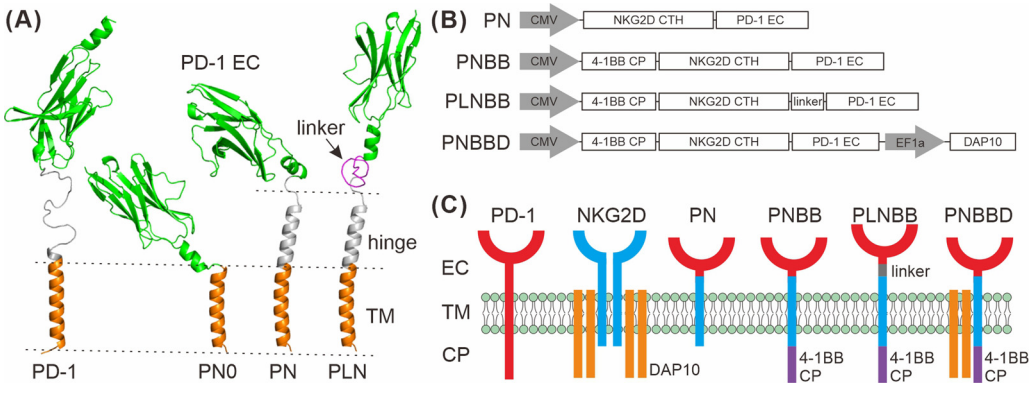

Fig. 1. Structure-based rational design and constructions of chimeric PD1-NKG2D (chPN) receptors. (**A**) 3D structures of transmembrane and ectodomains of wild-type PD-1 and chPN receptors. Models were constructed by PredMP and I-TASSER based on human PD-1 structure (Protein Data Bank: 5IUS). The ectodomain of PD-1, transmembrane domains of PD-1 and NKG2D, and hinge region of NKG2D are colored in green, orange, and gray, respectively, and the GS linker is colored in magenta. (**B**) Schematic representation of various chPN receptors. Each promoter is indicated by a gray arrow. (**C**) Schematic diagram of wild-type PD-1, NKG2D-DAP10 complex and various chPN receptors. Extracellular (EC), transmembrane (TM), cytoplasmic (CP) domains and GS linker are as indicated. The cytoplasmic domain, the transmembrane domain, and the hinge region of NKG2D are further abbreviated as CTH (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

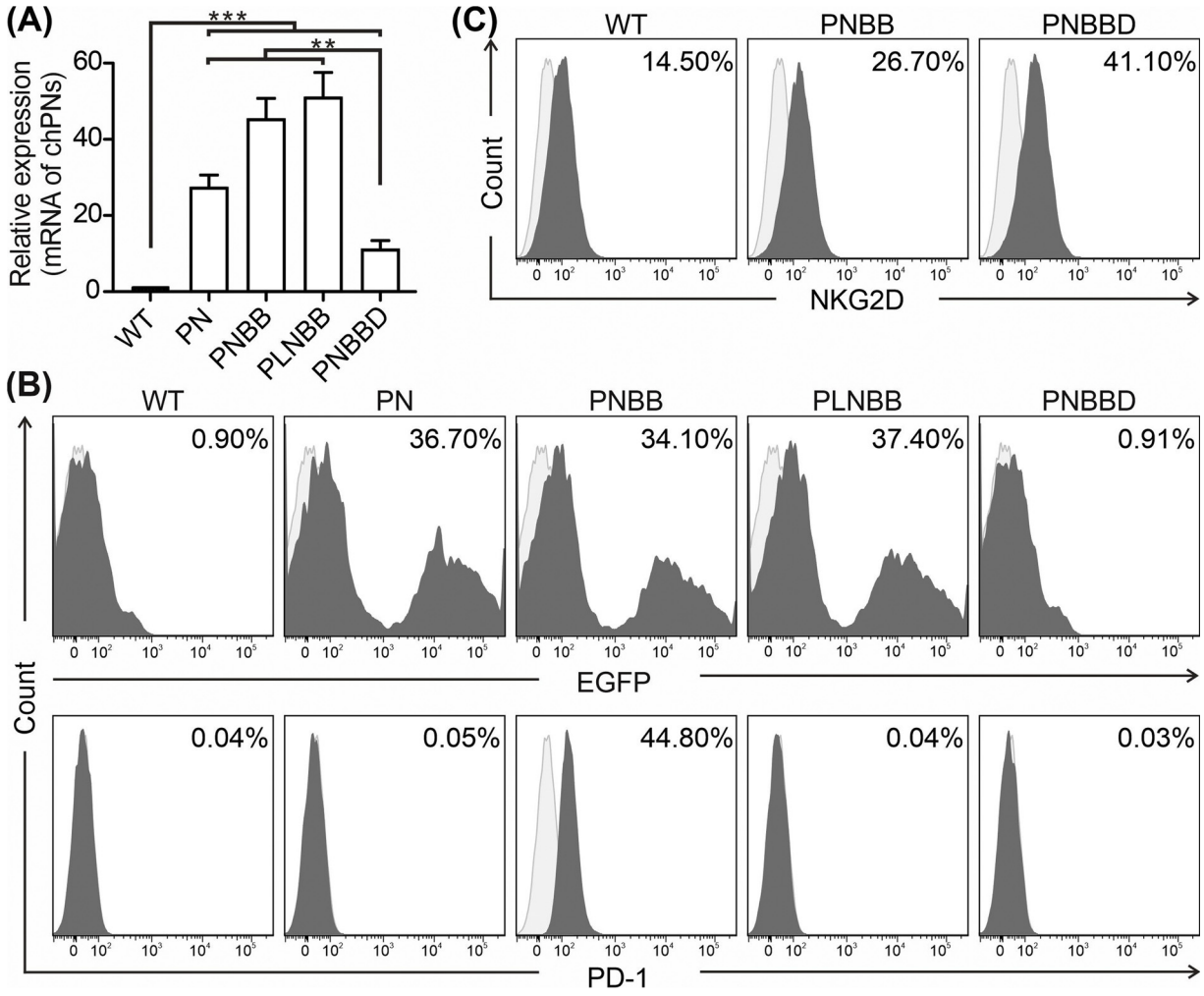

Fig. 2. Expression profiles of chPN receptors in various NK92 cells. (**A**) Relative transcriptional expression of chimeric receptor genes in untransduced (WT) and transduced NK92 cells. Data are shown as mean ± SD. Statistical analysis was done by two-tailed Student's *t*-test; *p < 0.05, **p < 0.01, ***p < 0.001. (**B-C**) Expression of EGFP and surface expression of PD-1 ectodomain (**B**) and NKG2D (**C**) in untransduced (WT) and transduced NK92 cells, as assessed by flow cytometry. NK92 cells untransduced or transduced by various chimeric PN receptors were stained with anti-PD1 mAbs and anti-NKG2D mAbs.

ectodomain surface expression (Fig. 2B, Table S1). These data indicated that the cytoplasmic domain of 4-1BB favored expression or stability of the chimeric PNBB protein. Thus, the difference in surface expression of chimeric receptors likely reflects differences in protein translation, stability, or turnover of the chPN receptors. For PNBBD-NK92 cells, exogenously expressed DAP10 improved surface expression of endogenous NKG2D by contributing to its stability (Fig. 2C, Table S1), but the surface expression of PNBBD was not improved (Fig. 2B). In this case, fusing the DAP10 intracellular domain to the N-terminus of PNBB may be appropriate.

3.3. *In vitro* cytotoxicity of PNBB-NK92 cells against tumor cells

The ligand for PD-1, PD-L1 is highly expressed in many types of human solid tumors. In order to identify chPN receptor-engineered NK92 cell therapeutic efficacy against PD-L1-expressing solid tumor cells, surface expression of PD-L1 on two human lung cancer cell lines A549 and H1299 was determined. The flow cytometry results demonstrated that A549 cells have limited PD-L1 expression, while H1299 cells have a significantly higher expression of PD-L1 (Fig. 3A, Table S2). Untransduced and chimeric receptor-transduced NK92 cells were co-cultured for 5 h with A549 cells or H1299 cells at an effector-to-target (E:T) ratio of 5:1. This optimal ratio was determined by the killing effect of PNBB-NK92 cells on tumor cell lines at different E:T ratios (Fig. S1).

Cytotoxicity assays showed that only the PNBB-NK92 cells displayed specific killing of H1299 cells but not A549 cells (Fig. 3B). These results indicated that the capability to kill tumor cells *in vitro* correlated with the cell surface expression of individual chPN receptors. The specific lysis of H1299 cells mediated by PNBB-NK92 cells was verified by high content cell imaging. After 1.5 h of incubation with PNBB-NK92 cells, most H1299 cells had been detached from the culture plate and eventually progressed to cellular swelling, rupture, and death (Fig. S2), which are typical features of pyroptosis (Zhang et al., 2018). This result indicated that the rapid clearance of cancer cells by PNBB-NK92 cells is likely to be mediated and promoted by the pyroptotic cell death pathway(Wang et al., 2017a). Next, we will clarify how PNBB-NK92 cells induced specific lysis of H1299 cells *via* pyroptosis pathway.

Both untransduced NK92 cells and PNBB-NK92 cells showed significant secretion of cytokines IFN-γ, TNF-α, granzyme B, and porforin when co-cultured with H1299 cells, but there was no significant difference between them (Fig. 3C). PNBB-NK92 cells produced much more reduced IFN-γ and TNF-α than CD19-CAR T cells (Ying et al., 2019), so PNBB-NK92 cells have a reduced risk of CRS and a higher safety. To examine whether PNBB-NK92 cells exhibit cytotoxicity against other tumor cells with PD-L1 expression, PNBB-NK92 cells were co-cultured with melanoma cell line SK-MEL-28, bone osteosarcoma cell line U2OS, and mammary carcinoma cell line $EMT6^{PDL1+}$ tumor cell lines at E:T

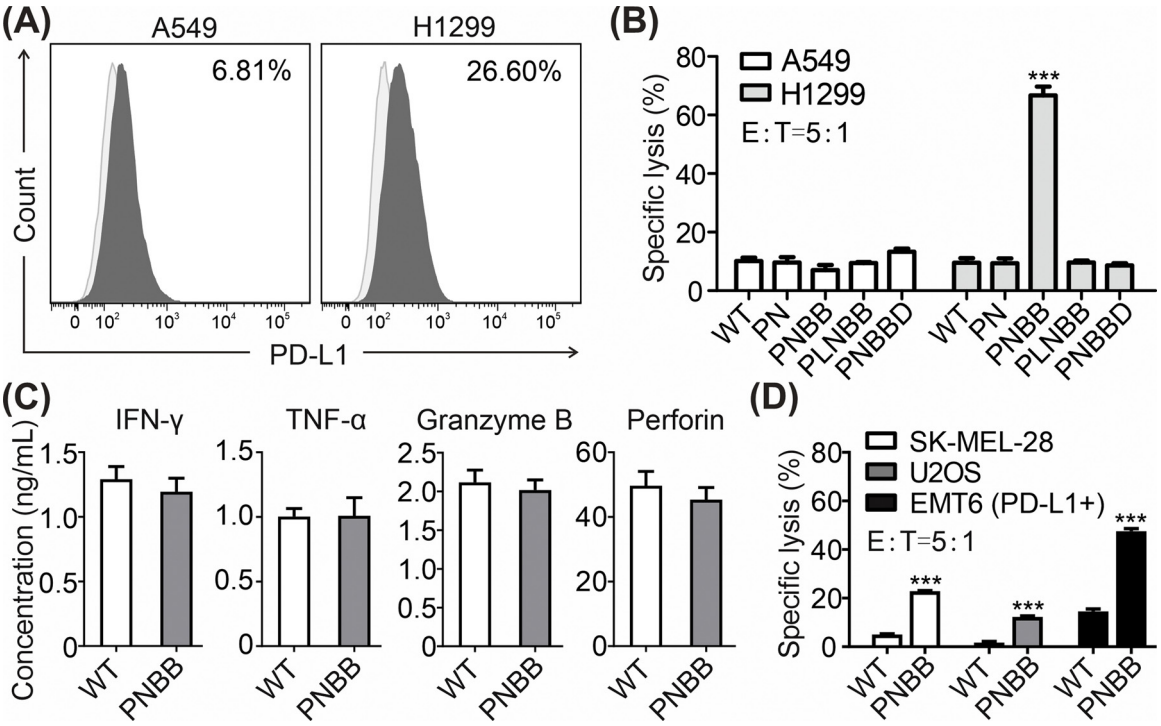

Fig. 3. Cytotoxicity activity and cytokine secretion of transduced NK92 cells. (**A**) Surface expression of PD-L1 in lung cancer cell lines A549 and H1299, as assessed by flow cytometry. (**B**) LDH release cytotoxicity assays were performed to measure the specific lysis of A549 and H1299 cells by untransduced (WT) or transduced NK92 cells. (**C**) Production of IFN-γ, TNF-α, granzyme B and porforin were assessed by ELISA in untransduced (WT) NK92 cells and PNBB-NK92 cells after co-culturing with H1299 tumor cells. (**D**) LDH release cytotoxicity assays measure specific lysis of tumor cells by untransduced (WT) NK92 cells and PNBB-NK92 cells. Data are shown as mean ± SD. Statistical analysis was done by two-tailed Student's *t*-test; ***$p < 0.001$.

ratios of 5:1. As expected, PNBB-NK92 cells demonstrated enhanced cytotoxicity against all tumor cell lines compared with untransfected NK92 cells (Fig. 3D).

4. Discussion

In this study, a native activating receptor, NKG2D was harnessed to provide a powerful and dominant activating effect on NK cells. We took PD-1 ectodomain and NKG2D transmembrane domain as a backbone to rationally design four chimeric receptors by transmembrane protein structure modeling. One of the chimeric receptors consisting of the NKG2D hinge region and 4-1BB signaling domain, PNBB exhibited stable surface expression on NK92 cells (Fig. 2) and mediated robust *in vitro* cytotoxicity against various tumor cells of NK92 cells (Fig. 3B). Therefore, this strategy now provides a promising approach for the rational design of NK cell-tailored chimeric receptors with NKG2D signaling.

CAR-engineered NK cells have the potential in the treatment of solid tumors (LeslieSep. 13 et al., 2018), but most CARs do not properly utilize the primary activation signaling pathway of NK cells (Siegler et al., 2018; Zhang et al., 2017). As a vital activating cytotoxicity receptor on NK cells, NKG2D is suitable as an ideal backbone for building NK cell-based CARs. Current NKG2D-based CARs utilize NKG2D extracellular domain rather than the signaling moiety to mediate T cells or NK cells to target tumor cells expressing NKG2D ligands (Han et al., 2018; Sentman and Meehan, 2014; Tao et al., 2018). Due to the odd properties of NKG2D, it is still a challenge for CAR design to activate NK cells only by the transmembrane domain of NKG2D.

First, the NKG2D receptor consists of a short cytoplasmic tail that lacks signaling properties, so it is quite confusing that Wang et al. used the cytoplasmic domain of NKG2D to construct CAR to mediate NK cell activation (Wang et al., 2017b). Second, most platforms of CARs are derived from type I transmembrane proteins with an extracellular N-terminus and intracellular C-terminus. Thus, when the transmembrane domain of the type II protein NKG2D is used as the backbone for CAR construction, conformational changes of other elements will be unavoidable. Li et al. constructed NKG2D-based CARs by forcedly reversing the orientation of NKG2D transmembrane domain by a signal peptide (Li et al., 2018). Indeed, this strategy can reduce the conformational interference of the NKG2D segment to other CAR components, but it is unclear whether the inverted NKG2D segment could interact with its transmembrane signaling adaptor protein DAP10. Therefore, there has been no persuasive case of building CARs by naïve NKG2D transmembrane domain rather than full-length NKG2D.

Here, we established an appropriate strategy based on protein structure modeling to design and construct NKG2D signaling-based chimeric receptors rationally. Due to their important role in the conformation and function of CARs (Ying et al., 2019), we genetically altered the sequences encoding the hinge and cytoplasmic domain to create a panel of representative variants of chimeric PD1-NKG2D. The short cytoplasmic tail of NKG2D was retained to buffer the impact on the 4-1BB costimulatory domain. Furthermore, the conformational change of PD-1 ectodomain caused by NKG2D transmembrane domain was assessed by protein structure modeling. The tertiary structures of chimeric proteins indicated that the hinge region of NKG2D ectodomain improved the elasticity of the PD-1 ectodomain, and the flexible GS linker (Chen et al., 2013; Yu et al., 2015) further provided better flexibility of PD-1 ectodomain (Fig. 1A).

However, the surface expression of most chimeric PD1-NKG2D receptors in this study was undetectable (Fig. 2B), which may be caused by the instability and transmembrane inefficiency of the chimeric proteins. Although sequence chimerization can alleviate the intrinsic instability of the NKG2D protein, this may be not enough to prevent the poor stability caused by NKG2D backbone. These results, which are beyond the design expectations, are the embodiment of the "design-build-test-redesign" cycle in synthetic biology research. Increasing the

expression of DAP10 protein by 2A self-cleaving peptides (Wang et al., 2015) to avoid transcription interference and reducing the length of the extracellular region by retaining the ligand-binding core of PD-1 in combination with short hinges (*e.g.* IgG4 hinge) (Chang et al., 2018) are probably superior solutions.

5. Conclusion

In conclusion, we showed how to use the NKG2D transmembrane signaling to build chimeric receptors by computer-aided rational design. A novel chimeric receptor PNBB with stable surface expression mediated robust *in vivo* cytotoxicity of NK92 cells against various tumor cells. The data in this study also suggest that rational design based on computational modeling and signaling optimization can help accelerate the "design-build-test-redesign" cycle and the development of potent functional NK-tailored CARs.

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Declaration of Competing Interest

The authors declare no conflicts of interest for this article.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.07.009.

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